

学校编码: 10384  
学号: 21620131152595

分类号\_\_密级\_\_  
UDC\_\_

廈門大學

碩 士 學 位 論 文

约氏疟原虫配子体特异表达基因的功能  
分析

Functional Analysis of Gametocyte-Specific Genes in the  
Rodent Malaria Parasite *Plasmodium yoelii*

凌渊

指导教师姓名: 袁晶教授

专 业 名 称: 生物化学与分子生物学

论文提交日期: 2016 年 4 月

论文答辩时间: 2016 年 5 月

学位授予日期: 2016 年 月

答辩委员会主席: \_\_\_\_\_

评 阅 人: \_\_\_\_\_

2016 年 月

# 厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果,均在文中以适当方式明确标明,并符合法律规范和《厦门大学研究生学术活动规范(试行)》。

另外,该学位论文为( )课题(组)的研究成果,获得( )课题(组)经费或实验室的资助,在( )实验室完成。(请在以上括号内填写课题或课题组负责人或实验室名称,未有此项声明内容的,可以不作特别声明。)

声明人(签名):

年 月 日

厦门大学博硕士论文摘要库

# 厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

（        ） 1. 经厦门大学保密委员会审查核定的保密学位论文，  
于        年        月        日解密，解密后适用上述授权。

（        ） 2. 不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年        月        日

厦门大学博硕士论文摘要库

## 摘要

疟疾是由疟原虫感染导致的蚊媒传染性疾病, 每年导致全球上亿人口的感染和上百万人口的死亡, 严重危害人类健康。疟原虫具有宿主交替和世代交替的特点, 有脊椎动物和按蚊两个宿主, 无性生殖和有性生殖两种生殖方式。配子体状态的疟原虫被按蚊吸食后, 先后经历雌雄配子体—雌雄配子—合子—动合子—卵囊等发育阶段, 这是一个多步骤、多基因调控的复杂过程, 是疟疾传播的必须阶段。迄今为止, 关于疟原虫配子体阶段特异表达基因的研究相对较少, 本研究将重点分析约氏疟原虫配子体阶段特异表达基因的生物学功能。

通过比较鼠伯氏疟原虫 ANKA 虫株五个发育阶段的转录谱(4h 环状体时期、16h 滋养体时期、22h 裂殖体时期、配子体时期及动合子时期) 和人恶性疟原虫 3D7 虫株七个发育阶段的转录谱(环状体时期、早期滋养体时期、晚期滋养体时期、裂殖体时期、配子体 II 期、配子体 V 期及动合子时期), 我们筛选出 18 个基因, 它们在配子体时期 mRNA 的表达量较之其它发育阶段均显著上调十倍以上, 呈现配子体阶段特异性表达。我们在鼠约氏疟原虫 17XNL 虫株上找到了这 18 个基因的同源基因, 命名为 G1-G18。

本研究以鼠约氏疟原虫 17XNL 为模型, 通过反向遗传学策略, 采用 CRISPR/Cas9 基因修饰技术, 构建 G1-G18 基因敲除载体, 并通过 47 次独立电击转染, 23 次单克隆, 制备 G1-G18 单基因敲除虫株。结果表明, 共有 17 个基因可以实现基因敲除, 并通过有限稀释法获得单基因敲除型单克隆虫株, 说明这些基因的功能对于疟原虫在红细胞阶段的发育是非必须的, 或者它们的功能能够被其它基因代偿; G3 基因设计了多个打靶位点, 电击转染 11 次, 但依然未获得基因敲除型单克隆虫株, 推测 G3 基因是疟原虫红细胞阶段生长发育的必须基因, 发挥重要作用。

对获得的 17 个基因的单基因敲除虫株进行功能分析, 选择了疟原虫生活周期中三个关键的发育节点, 分别是: 疟原虫在小鼠体内诱导生成雌雄配子体的能力(包括雌雄配子体率和雌雄配子体性别比例)、疟原虫蚊期动合子生成能力(包括动合子转化率和动合子正常形态比率)和疟原虫蚊期卵囊发育能力

(按蚊感染后第 6-7 天的卵囊数量)。

盐酸苯胍诱导小鼠体内疟原虫配子体生成实验和体外诱导疟原虫动合子生成实验的结果表明,这 17 个基因的单基因敲除,不影响疟原虫雌雄配子体生成和动合子的正常发育;

为了分析基因敲除虫株的潜在表型,我们进一步开展了这 17 个基因的单基因敲除虫株的按蚊感染实验。结果显示,所有单基因敲除虫株在感染按蚊后能够形成正常数量范围的卵囊(感染后第 6-7 天),与野生型虫株相比没有显著差异。

综上所述,本研究筛选出 18 个约氏疟原虫配子体时期特异表达的基因(G1-G18),构建了 G1-G18 的基因敲除载体,获得了 17 个基因的单基因敲除型单克隆虫株,初步探讨了配子体时期特异表达基因的生物学功能,结果表明,本研究中的 17 个基因对疟原虫雌雄配子体、动合子和卵囊的发育没有影响。

**关键词:** 疟原虫; 基因敲除; 配子体阶段特异表达基因

## Abstract

Malaria is the mosquito-transmitted infectious disease caused by *Plasmodium* parasite. Almost one million people died from it and billions of people infected with malaria annually, endangering human health. The *Plasmodium* parasite resides in the alternative vertebrate and mosquito host, transitioning between asexual and sexual forms. Parasite gametocytes were transmitted to mosquito midgut within blood meal, after which parasites undergo several processes including gametocyte, gamete, zygote, ookinete and oocyst development successively. The parasite development in the mosquito midgut is indispensable for malaria transmission, which is still poor understood mechanically considering its regulation via multi-steps and multi-genes.

This study mainly focused on the functional role of some gametocyte-specific genes in parasite development.

By comparing the parasite transcriptome in PlasmoDB database, including five different stages in rodent malaria parasite *Plasmodium berghei* ANKA strain (4h ring, 16h trophozoite, 22h schizont, gametocyte, ookinete) and seven stages in human malaria parasite *Plasmodium falciparum* 3D7 strain (ring, early trophozoite, late trophozoite, schizont, gametocyte II, gametocyteV, ookinete), we screened for genes with transcription at gametocyte-specific manner and chosen 18 genes for subsequent study. The corresponsive gene orthologous in another rodent malaria parasite *Plasmodium yoelii* 17XNL were obtained and named as G1-G18 genes.

Using reverse genetics strategy, gene deletion for G1-G18 was conducted via CRISPR/Cas9 method in *Plasmodium yoelii* 17XNL strain. In total, 47 independent electroporation transfection effort and subsequent 23 single cloning of gene deleted parasite culture were performed. As a result, gene deletions were successful achieved in 17 genes, suggesting that these genes are not essential to the parasite development in erythrocytic stage or the function of these genes can be



compensated with others. At least two independent cloned parasite strains for each gene deletion were obtained and tested for phenotype. However, G3 did not appear any efficiency with several target sites and eleven times electroporation transfection, suggesting G3 plays an important role in the parasite development in erythrocytic stage.

To understand the function of 17 genes, three key stages in parasite life cycle were assessed: gametocyte generation(gametocytemia and sex ratio), ookinete transformation(ookinete conversion rate and correct cell shape rate) and oocyst development(oocyst number on day 6-7 post-blood feeding).

It was demonstrated that there was no significant difference between 17 deletion mutants and wild type strain on gametocyte formation or ookinete differentiation when gametocytogenesis and ookinete conversion were assessed.

To further analyze the phenotype, mosquitoes were fed on mice infected with wild type or knockout mutants parasite. It showed that oocyst number on day 6-7 post-infection was comparable to that of wild type parasites.

In conclusion, our study identified 18 gametocyte-specific genes in *Plasmodium yoelii* 17XNL strain and produced 17 gene deletion mutants. Analysis of these mutants identified no phenotypic differences from wild type parasite during gametocyte, ookinete and oocyst development.

**Keywords:** *Plasmodium* parasite; gene knockout; gametocyte-specific genes

# 目录

摘要.....	I
Abstract .....	III
目录.....	V
Contents .....	VIII
第一章 前言.....	1
1.1 疟疾和疟原虫.....	1
1.2 疟原虫生活史 (Life Cycle) .....	2
1.3 疟原虫在按蚊中肠内的生殖发育 .....	4
1.4 啮齿类动物疟原虫 .....	6
1.5 疟原虫配子体阶段特异表达基因 (gametocyte-specific) 研究进展 .....	7
1.5.1 P47、P48/45 和 P230 在雌雄配子受精过程中起到关键作用.....	7
1.5.2 Pfg377 对雌配子体嗜饿小体的形成和雌配子从红细胞中的释放过程发挥重要作用 .....	8
1.5.3 MDV-1/PEG3 介导雌雄配子从红细胞的释放 .....	8
1.5.4 gABCG2 在疟原虫脂代谢过程中发挥重要作用 .....	9
1.6 本研究的目的与意义 .....	9
第二章 约氏疟原虫配子体时期特异表达基因的筛选 .....	11
2.1 引言 .....	11
2.2 基因筛选策略 .....	12
2.3 基因筛选结果 .....	12
2.4 小结及讨论 .....	16
第三章 约氏疟原虫配子体时期特异表达基因的单基因敲除虫株构建 .....	17
3.1 引言 .....	17
3.2 实验材料.....	17
3.3 实验方法.....	21
3.3.1 虫株感染、冻存和复苏 .....	21
3.3.2 血涂片姬姆萨染色 .....	21
3.3.3 疟原虫高纯度基因组 DNA 提取 .....	21
3.3.4 疟原虫基因组 DNA 快速提取 .....	21
3.3.5 约氏疟原虫电击转染步骤 .....	22
3.3.6 有限稀释法获得单克隆虫株 .....	23
3.3.7 基于 CRISPR/Cas9 系统构建打靶载体.....	23
3.3.7.1 克隆左右同源臂 (LA-left arm, RA-right arm) .....	23
3.3.7.2 左同源臂连入 SV 载体中: SV-LA.....	24
3.3.7.3 右同源臂连入载体 SV-LA 中: SV-LRA.....	25
3.3.7.4 克隆含打靶位点 sgRNA 的 pYC 载体: pYC-TS.....	25
3.3.7.5 构建同时含有 sgRNA 序列和左右同源臂的终载体: pYC-TLR.....	26
3.3.8 电击转染获得单基因敲除虫株 .....	27
3.3.8.1 电击转染 .....	27
3.3.8.2 加药筛选 .....	27

3.3.8.3 快提基因组鉴定打靶位点是否发生同源重组 .....	27
3.3.8.4 单克隆 .....	27
3.4 实验结果 .....	27
3.4.1 CRISPR/Cas9 系统介导的 G1 基因敲除 .....	29
3.4.1.1 G1 基因敲除原理示意图 .....	29
3.4.1.2 G1 基因敲除效率鉴定 .....	29
3.4.1.3 G1 基因敲除型单克隆虫株鉴定 .....	30
3.4.2 CRISPR/Cas9 介导的 G2 基因敲除 .....	31
3.4.3 CRISPR/Cas9 介导的 G3 基因敲除 .....	31
3.4.4 CRISPR/Cas9 介导的 G4 基因敲除 .....	32
3.4.5 CRISPR/Cas9 介导的 G5 基因敲除 .....	32
3.4.6 CRISPR/Cas9 介导的 G6 基因敲除 .....	33
3.4.7 CRISPR/Cas9 介导的 G7 基因敲除 .....	33
3.4.8 CRISPR/Cas9 介导的 G8 基因敲除 .....	34
3.4.9 CRISPR/Cas9 介导的 G9 基因敲除 .....	34
3.4.10 CRISPR/Cas9 介导的 G10 基因敲除 .....	35
3.4.11 CRISPR/Cas9 介导的 G11 基因敲除 .....	35
3.4.12 CRISPR/Cas9 介导的 G12 基因敲除 .....	36
3.4.13 CRISPR/Cas9 介导的 G13 基因敲除 .....	36
3.4.14 CRISPR/Cas9 介导的 G14 基因敲除 .....	37
3.4.15 CRISPR/Cas9 介导的 G15 基因敲除 .....	37
3.4.16 CRISPR/Cas9 介导的 G16 基因敲除 .....	38
3.4.17 CRISPR/Cas9 介导的 G17 基因敲除 .....	38
3.4.18 CRISPR/Cas9 介导的 G18 基因敲除 .....	39
3.5 小结与分析 .....	40
第四章 约氏疟原虫配子体特异表达基因的功能分析 .....	42
4.1 引言 .....	42
4.2 实验材料 .....	43
4.3 实验方法 .....	44
4.3.1 盐酸苯肼 (PHZ) 诱导配子体大量生成 .....	44
4.3.2 动合子体外诱导转化 .....	44
4.3.3 血饲按蚊感染 .....	45
4.3.4 按蚊卵囊红汞染色 .....	47
4.3.5 相关实验计算公式 .....	47
4.3.5.1 原虫率 (Parasitemia) .....	47
4.3.5.2 配子体率 (Gametocytemia) .....	47
4.3.5.3 动合子转化率 (Ookinete Conversion Rate) 和动合子正常形态比率 (Correct Cell Shape Rate) .....	48
4.4 实验结果 .....	48
4.4.1 G1 基因敲除单克隆虫株表型分析 .....	50
4.4.2 G2 基因敲除单克隆虫株表型分析 .....	51
4.4.3 G4 基因敲除单克隆虫株表型分析 .....	52
4.4.4 G5 基因敲除单克隆虫株表型分析 .....	53

4.4.5 G6 基因敲除单克隆虫株表型分析 .....	54
4.4.6 G7 基因敲除单克隆虫株表型分析 .....	55
4.4.7 G8 基因敲除单克隆虫株表型分析 .....	56
4.4.8 G9 基因敲除单克隆虫株表型分析 .....	57
4.4.9 G10 基因敲除单克隆虫株表型分析 .....	58
4.4.10 G11 基因敲除单克隆虫株表型分析 .....	59
4.4.11 G12 基因敲除单克隆虫株表型分析 .....	60
4.4.12 G13 基因敲除单克隆虫株表型分析 .....	61
4.4.13 G14 基因敲除单克隆虫株表型分析 .....	62
4.4.14 G15 基因敲除单克隆虫株表型分析 .....	63
4.4.15 G16 基因敲除单克隆虫株表型分析 .....	64
4.4.16 G17 基因敲除单克隆虫株表型分析 .....	65
4.4.17 G18 基因敲除单克隆虫株表型分析 .....	66
4.5 小结与分析 .....	67
第五章 总结与讨论 .....	68
5.1 总结 .....	68
5.2 CRISPR/Cas9 基因修饰技术 .....	68
5.2.1 CRISPR/Cas9 基因修饰技术存在脱靶效应 .....	69
5.2.2 降低 CRISPR/Cas9 基因修饰技术脱靶效应的策略 .....	69
5.3 疟原虫中存在翻译抑制现象 .....	70
参考文献 .....	72
附图 .....	76
附表 .....	78
附表 1. CRISPR/Cas9 基因敲除分子克隆同源重组模板扩增引物 .....	78
附表 2. sgRNA 靶向序列 .....	79
附表 3. 同源重组及单克隆效率鉴定引物 .....	80
附表 4. 缩略语中英文对照 .....	81
图表索引 .....	83
攻读学位期间发表论文 .....	85
致谢 .....	86

厦门大学博硕士论文摘要库

## Contents

Abstract in Chinese .....	I
Abstract in English .....	III
Contents in Chinese .....	V
Contents in English .....	VIII
Chapter 1: Introduction .....	1
1.1 Malaria and <i>Plasmodium</i> .....	1
1.2 Life cycle of the <i>Plasmodium</i> parasite .....	2
1.3 Developments of <i>Plasmodium</i> in <i>Anopheles</i> mosquito midgut .....	4
1.4 Rodent malaria parasite .....	6
1.5 Research progresses on <i>Plasmodium</i> gametocyte-specific genes .....	7
1.5.1 P47、P48/45 and P230 play an important role in gametocyte fertilization .....	7
1.5.2 Pfg377 is associated with the production of osmiophilic body and the emergence of female gamete .....	8
1.5.3 Egress of male and female gametes from their host erythrocyte is mediated by the MDV-1/PEG3 protein .....	8
1.5.4 gABCG2 plays a role in lipid metabolism in the malaria parasite .....	9
1.6 The purpose and significance of this research .....	9
Chapter II: Screening of gametocyte-specific genes in <i>Plasmodium yoelii</i> .....	11
2.1 Introduction .....	11
2.2 Strategy to screen target genes .....	12
2.3 Genetic screening results .....	12
2.4 Summary and analysis .....	16
Chapter III: Construction of single gene knockout mutants in <i>Plasmodium yoelii</i> gametocyte-specific genes .....	17
3.1 Introduction .....	17
3.2 Experimental materials .....	17
3.3 Experimental methods .....	21
3.3.1 Infection、cryopreservation and recovery of <i>Plasmodium</i> parasite .....	21
3.3.2 Gimsa-staining .....	21
3.3.3 DNA extraction of parasite with high quality .....	21
3.3.4 Quick extraction of parasite DNA .....	21
3.3.5 <i>Plasmodium yoelii</i> electroporation steps .....	22
3.3.6 Dilution method of monoclonal strain .....	23
3.3.7 Construction of target vectors based on CRISPR/Cas9 system .....	23
3.3.7.1 HDR template cloning (LA-left arm, RA-right arm) .....	23
3.3.7.2 Ligation of left arm into SV vector: SV-LA .....	24
3.3.7.3 Ligation of right arm into SV-LA vector: SV-LRA .....	25
3.3.7.4 Cloning pYC with target sequence sgRNA: pYC-TS .....	25
3.3.7.5 Construction of the final vector containing both sgRNA and HDR template: pYC-TLR .....	26

3.3.8 Obtain of single gene knockout mutants via electroporation transfection .....	27
3.3.8.1 Electroporation transfection.....	27
3.3.8.2 Screening through drug selection.....	27
3.3.8.3 PCR detection of knockout mutants .....	27
3.3.8.4 Single cloning.....	27
3.4 Results .....	27
3.4.1 G1 knockout based on CRISPR/Cas9 system .....	29
3.4.1.1 Schematic diagram of G1 knockout strategy.....	29
3.4.1.2 PCR detection of knockout efficiency of G1 .....	29
3.4.1.3 PCR detection of knockout single clone efficiency of G1 .....	30
3.4.2 G2 knockout based on CRISPR/Cas9 system .....	31
3.4.3 G3 knockout based on CRISPR/Cas9 system .....	31
3.4.4 G4 knockout based on CRISPR/Cas9 system .....	32
3.4.5 G5 knockout based on CRISPR/Cas9 system .....	32
3.4.6 G6 knockout based on CRISPR/Cas9 system .....	33
3.4.7 G7 knockout based on CRISPR/Cas9 system .....	33
3.4.8 G8 knockout based on CRISPR/Cas9 system .....	34
3.4.9 G9 knockout based on CRISPR/Cas9 system .....	34
3.4.10 G10 knockout based on CRISPR/Cas9 system .....	35
3.4.11 G11 knockout based on CRISPR/Cas9 system .....	35
3.4.12 G12 knockout based on CRISPR/Cas9 system .....	36
3.4.13 G13 knockout based on CRISPR/Cas9 system .....	36
3.4.14 G14 knockout based on CRISPR/Cas9 system .....	37
3.4.15 G15 knockout based on CRISPR/Cas9 system .....	37
3.4.16 G16 knockout based on CRISPR/Cas9 system .....	38
3.4.17 G17 knockout based on CRISPR/Cas9 system .....	38
3.4.18 G18 knockout based on CRISPR/Cas9 system .....	39
3.5 Summary and analysis .....	40
Chapter IV: Functional analysis of gametocyte-specific genes in the rodent malaria parasite <i>Plasmodium yoelii</i> .....	42
4.1 Introduction .....	42
4.2 Experimental materials.....	43
4.3 Experimental methods .....	44
4.3.1 Inducing the production of more gametocytes by PHZ .....	44
4.3.2 Ookinete conversion in vitro.....	44
4.3.3 Blood meal.....	45
4.3.4 Oocyst mercurochrome staining .....	47
4.3.5 Some relative formulas.....	47
4.3.5.1 Parasitemia .....	47
4.3.5.2 Gametocytemia .....	47
4.3.5.3 Ookinete conversion rate and correct cell shape rate .....	48
4.4 Results .....	48
4.4.1 Phenotypic analyses of G1 mutants .....	50

4.4.2 Phenotypic analyses of G2 mutants .....	51
4.4.3 Phenotypic analyses of G4 mutants .....	52
4.4.4 Phenotypic analyses of G5 mutants .....	53
4.4.5 Phenotypic analyses of G6 mutants .....	54
4.4.6 Phenotypic analyses of G7 mutants .....	55
4.4.7 Phenotypic analyses of G8 mutants .....	56
4.4.8 Phenotypic analyses of G9 mutants .....	57
4.4.9 Phenotypic analyses of G10 mutants .....	58
4.4.10 Phenotypic analyses of G11 mutants .....	59
4.4.11 Phenotypic analyses of G12 mutants .....	60
4.4.12 Phenotypic analyses of G13 mutants .....	61
4.4.13 Phenotypic analyses of G14 mutants .....	62
4.4.14 Phenotypic analyses of G15 mutants .....	63
4.4.15 Phenotypic analyses of G16 mutants .....	64
4.4.16 Phenotypic analyses of G17 mutants .....	65
4.4.17 Phenotypic analyses of G18 mutants .....	66
4.5 Summary and analysis .....	67
Chapter V: Conclusions and discussions.....	68
5.1 Conclusions.....	68
5.2 CRISPR/Cas9 system .....	68
5.2.1 Off-target of CRISPR/Cas9 system .....	69
5.2.2 Strategies to lower the occurrence of off-target caused by CRISPR/Cas9 .....	69
5.3 Translational Repression in <i>Plasmodium</i> .....	70
Reference .....	72
Supplemental figure .....	76
Supplemental tables.....	78
Table S1. Primers of homologous recombination template cloning based on CRISPR/Cas9 gene knockout .....	78
Table S2. Target sgRNA sequences.....	79
Table S3. Primers of homologous recombination detection and knockout single cloning detection.....	80
Table S4. Abbreviations list .....	81
Index of figures and tables .....	83
Publications .....	85
Acknowledgements .....	86



Degree papers are in the “[Xiamen University Electronic Theses and Dissertations Database](#)”.

Fulltexts are available in the following ways:

1. If your library is a CALIS member libraries, please log on <http://etd.calis.edu.cn/> and submit requests online, or consult the interlibrary loan department in your library.
2. For users of non-CALIS member libraries, please mail to [etd@xmu.edu.cn](mailto:etd@xmu.edu.cn) for delivery details.